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Use of *lacZ* Fusions To Measure In Vivo Expression of the First Three Genes of the *Escherichia coli unc* Operon

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We have constructed in-frame *lacZ* protein fusions to the first three genes of the *Escherichia coli unc* operon, which codes for the subunits of the proton-translocating ATPase. We have used these constructions to measure the relative in vivo expression of these genes. The second and third genes, *uncB* and *uncE*, which code for the a and c subunits of the F_0 sector, were expressed at relative levels of approximately 1:10, although the measured expression of *uncB* depended upon how much of the gene was fused to *lacZ*. These rates compared favorably with the relative numbers of a and c subunits (a_1:c_{10}) in the purified F_1F_0 complex. The in vivo expression of *uncI*, the first gene of the operon, was very low, at best 10 to 20 times less than the expression of *uncB*.

The proton-translocating ATP synthase of *Escherichia coli* couples an electrochemical gradient of protons to the synthesis of ATP from ADP and P_i. Under anaerobic conditions, the enzyme functions in the reverse direction as an ATPase, hydrolyzing ATP and pumping protons across the cytoplasmic membrane to form a proton gradient. This gradient is used to carry out many cellular processes, including the essential uptake of nutrients such as sugars and amino acids.

The ATPase consists of two sectors, the F_0 and the F_1. The F_0 sector contains three subunits, designated a, b, and c, which are assembled as an integral part of the membrane. This sector binds F_1 to the membrane and provides a channel through which protons are translocated. The F_1 sector, which is composed of five subunits, α, β, γ, δ, and ε, is bound to the F_0 and carries the catalytic sites for ATP synthesis and hydrolysis (10, 21, 22).

The genes which code for the eight subunits of the ATPase are contained within the *unc* operon, located at 83.5 min on the *E. coli* chromosome. The *unc* operon actually consists of nine genes which are transcribed in the order *uncI*, *-B*, *-E*, *-F*, *-H*, *-A*, *-G*, *-D*, and *-C*, corresponding to protein i and subunits a, c, b, δ, α, γ, β, and ε, respectively. The role of protein i is currently not known, but it has been shown that this protein is not required for the activity or biosynthesis of the ATPase complex (11, 28).

Each gene exists in a single copy within the operon, and the operon is transcribed into a single polycistronic mRNA, yet the subunits encoded by these genes exist in different numbers in the assembled complex. Particularly intriguing is the stoichiometry of the a and c subunits of the F_0 sector, which has been determined to be 1:10 (7). This unusual stoichiometry raises questions about the regulation of gene expression. Results from studies on the synthesis of ATPase polypeptides in vivo using minicells, in vitro in a transcription-translation system, or in UV-irradiated λ *unc* lysogens indicate that the a and c subunits are synthesized differentially (4, 18). It appears that in order for the subunits to be synthesized in the appropriate relative amounts, there must be some sort of regulation, probably at the level of translation, which would allow for the expression of *uncE* to increase over that of *uncB*. The exact mechanism of control over the apparent differences in expression exhibited by these genes is currently unknown. McCarthy and coworkers, however, have demonstrated that an RNA sequence within the translational initiation region of *uncE* causes increased synthesis of the c subunit (15) and can also increase the expression of other genes when located within the translation initiation region of those genes (16). This sequence, therefore, appears to enhance expression of *uncE*.

This research was aimed at quantitating the in vivo expression of the first three genes of the *E. coli unc* operon. A series of *lacZ* protein fusions were constructed in *uncI*, *uncB*, and *uncE*, and the relative level of expression of each gene was determined by comparing the amount of β-galactosidase activity obtained from the corresponding fusion proteins. All fusions contained the *unc* promoter, to drive the transcription of the cloned genes, and *uncI*, the first gene of the *unc* operon. Four fusions were made in *uncB* to test the effect of the amount of *uncB* DNA fused to *lacZ* on β-galactosidase activity.

MATERIALS AND METHODS

Media and chemicals. LB medium (10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract per liter) was used for bacterial growth (17). Minimal A medium consisted of 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, and 0.5 g of sodium citrate per liter, 1 mM $MgSO_4$, 0.4% glucose, and 10 μg of B1 per liter. TBM medium consisted of 10 g of tryptone per liter, 5 g of NaCl per liter, and 0.2% maltose. Antibiotic was added to the media in the following concentration: ampicillin, 30 mg/liter. The Lac^+ phenotype was screened for either on LB-ampicillin plates containing the chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) or on MacConkey agar plates containing ampicillin.

Bacteria, bacteriophages, and plasmids. All bacterial strains, bacteriophages, and plasmids used in these studies are listed in Table 1.

Assay for β-galactosidase activity. β-Galactosidase assays were performed as described by Miller (17). Lysogens were grown to an optical density at 600 nm (OD_{600}) of 0.45 to 0.5 in 5 ml of minimal A medium. Portions of the culture (0.1 to 0.5 ml) were then added to the assay medium (0.1 M sodium phosphate [pH 7.0], 0.001 M $MgSO_4$, 0.2 mM $MnSO_4$, 0.05 M β-mercaptoethanol) to give a final volume of 1 ml. To

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TABLE 1. Genotypes of bacterial strains, bacteriophages, and plasmids

Strain, plasmid, or phage	Genotype or relevant genes carried	Origin or reference
Bacterial strains		
MC1000	F⁻ *araD139* Δ(*araABC-leu*)*7679 galU galK* Δ(*lac*)X74 *rpsL thi*	6
MC1000Δ(*uncI-uncC*)	MC1000 Δ(*uncI-uncC*)	1
LE392	F⁻ *supF supE hsdR galK trpR metB lacY tonA*	23
Plasmids		
pMLB524	pBR322 Δ(Tet^r) *lac'ZY'*	23
pMLB1034	pBR322 Δ(Tet^r) *lac'ZY'*	23
pMLB1069	pBR322 Δ(Tet^r) *lac'ZY'*	23
pWSB18	*uncI*$^+$ *B'*	5
pUC8	*lacI lacZ'*	26
pRPG45	*uncB*$^+$*E*$^+$*F*$^+$*H*$^+$	13
pRPG58	*uncE*$^+$	13
pEN711	Φ(*uncBE'-'lacZ*)*1*(Hyb)	This study
pKS100	Φ(*uncI'-'lacZ*)*1*(Hyb)	This study
pKS101	Φ(*uncI'-'lacZ*)*2*(Hyb)	This study
pKS102	Φ(*uncIB'-'lacZ*)*1*(Hyb) (60 bases of *uncB*)	This study
pDKWH103	Φ(*uncIB'-'lacZ*)*2*(Hyb) (92 bases of *uncB*)	This study
pKS103	Φ(*uncIB'-'lacZ*)*3*(Hyb) (126 bases of *uncB*)	This study
pKS104	Φ(*uncIB'-'lacZ*)*4*(Hyb) (704 bases of *uncB*)	This study
pKS105	Φ(*uncIBE'-'lacZ*)*1*(Hyb)	This study
pKS113	pUC8 *uncI*$^+$ *B'*	This study
Phage		
λRZ5		12, 19
λC17*cI*$^-_{90}$	C17 *cI*$^-_{90}$	25
λpKS101	Φ(*uncI'-'lacZ*)*1 bla*	This study
λpKS102	Φ(*uncIB'-'lacZ*)*1 bla*	This study
λpDKWH103	Φ(*uncIB'-'lacZ*)*2 bla*	This study
λpKS103	Φ(*uncIB'-'lacZ*)*3 bla*	This study
λpKS104	Φ(*uncIB'-'lacZ*)*4 bla*	This study
λpKS105	Φ(*uncIBE'-'lacZ*)*1 bla*	This study

permeabilize the cells, 2 drops of $CHCl_3$ and 1 drop of 0.1% sodium dodecyl sulfate (SDS) were added to the sample. The sample was then mixed and placed in a 28°C water bath. The reaction was initiated after 5 min of incubation by addition of 0.2 ml of *o*-nitrophenyl-β-D-galactoside (ONPG); 4 mg/ml in 0.25 M sodium phosphate [pH 7.0]). After sufficient yellow color had developed, the reaction was stopped by addition of 0.5 ml of 1 M Na_2CO_3. OD_{420} and OD_{550} were then determined. The total amount of β-galactosidase units was calculated by the following formula: units = $\{1{,}000 \times [OD_{420} - (1.75 \times OD_{550})]\}/(t \times v \times OD_{600})$, where t is length of time of assay in minutes and v is volume of culture used per milliliter of assay mixture.

Plasmid construction. To construct an *uncI'-'lacZ* fusion, plasmid pWSB18 (5) was digested with *Mlu*I and *Bam*HI, generating a 700-base-pair fragment extending from 96 bases preceding the *unc* promoter to 86 bases in *uncB*. This fragment was cloned into pUC8 which had been cut with *Sma*I and *Bam*HI. The resulting plasmid, pKS113, was then digested with *Eco*RI and *Hin*dIII. This digestion produced a 261-base-pair fragment that contained the *unc* promoter and 48 base pairs of DNA at the 5' end of *uncI*. This fragment was cloned into the *lacZ* fusion plasmid pMLB1069, which had been cut with *Eco*RI and *Hin*dIII, to create plasmid pKS100. The 199-base-pair *Hin*dIII fragment within *uncI* was then cloned from pKS113 into pKS100 which had been digested with *Hin*dIII. The resultant 6.5-kilobase-pair plasmid, designated pKS101, contained the *unc* promoter and 248 base pairs of *uncI* fused in frame to *'lacZ*. This gene fusion is designated I.

Four different *uncIB'-'lacZ* fusions were constructed. The first, pKS102, was constructed by digesting pWSB18 with *Mlu*I, treating the digested DNA with Klenow enzyme (DNA polymerase I large fragment) to fill in the ends, and then redigesting that DNA with *Pvu*II. The resultant 671-base-pair fragment, containing the *unc* promoter, *uncI*, and 60 bases of *uncB*, was cloned into *Sma*I-digested pMLB1034 to produce an in-frame *uncB'-'lacZ* fusion. This gene fusion is designated B_1.

A second *uncIB'-'lacZ* fusion, pKS103, was constructed by cloning the 670-base-pair *Ssp*I fragment from pWSB18 into *Sma*I-digested pMLB1034 to generate an in-frame *uncB'-'lacZ* fusion containing the *unc* promoter, *uncI*, and 127 base pairs of *uncB*. This gene fusion is designated B_3.

A third *uncIB'-'lacZ* fusion plasmid, pDKWH103, was constructed by digesting pKS103 with *Bam*HI and religating, thus deleting 42 bases and producing an in-frame fusion containing 92 bases of *uncB* from the start codon to the *Bam*HI site. This gene fusion is designated B_2.

The last *uncIB'-'lacz* fusion, pKS104, was made at the *Bam*HI site near the 3' end of *uncB*. The *uncB* gene contains two *Bam*HI sites, separated by 617 base pairs. Digesting pKS103 with *Bam*HI and adding this 617-base-pair fragment would result in an out-of-frame fusion. We therefore digested pKS103 with *Bam*HI, treated the DNA with S1 nuclease to create blunt ends, and then cloned a linker (GATCCTAGGATCC) into the blunt-ended site so that after redigestion with *Bam*HI and insertion of the 617-base-pair *Bam*HI fragment described above, we would obtain an in-frame *uncB'-'lacZ* fusion containing the *unc* promoter, *uncI*, and 704 base pairs of *uncB*. This gene fusion is designated B_4.

The *uncIBE'-'lacZ* fusion was constructed from an *uncE'-'lacZ* fusion created with λ p*lac*Mu3. This phage, which has been described previously (3), was used to make an in-frame *uncE'-'lacZ* fusion in plasmid pRPG58 (13), which was transferred into plasmid pMLB524 (23). The fusion gene in the resultant plasmid, pEN711, contained 127 base pairs of *uncE*, 117 base pairs from the S end of phage Mu, and *'lacZ*. The fusion joint and the Mu region were both sequenced to ensure that the fusion was in frame. The *unc* promoter, *uncI*, and *uncB* were added to the plasmid by replacing the *Pst*I fragment extending from the *bla* gene of the vector into *uncB* with the 1,811-base-pair *Pst*I fragment from pKS103. The resultant plasmid, pKS105, contained the *unc* promoter, *uncI*, *uncB*, and the *uncE'-'lacZ* fusion described above. This gene fusion is designated E.

Moving fusion genes into the *E. coli* chromosome. The *unc'-'lacZ* fusion plasmids were transferred onto the transducing phage λRZ5 by infecting plasmid-bearing *E. coli* with the phage. A percentage of the phage in the resultant lysate had undergone a double recombination with the plasmid to incorporate all of the DNA between *bla* and *lacZ* onto the phage. Since the phage carry both *lac* and *bla*, they can be detected by infecting Lac⁻ *E. coli* and selecting for Ap^r Lac$^+$ cells, which have integrated the phage into the bacterial chromosome. The use of λRZ5 to move fusion genes from plasmids into the chromosome has been described previously (1, 12, 19). The fusions were directed to integrate at *att* by lysogenizing an *unc* deletion of *E. coli* MC1000.

Test for single lysogens. Since Lac$^+$ Ap^r phage that integrate into the chromosome can exist as single or multiple prophage forms, we tested each lysogen to ensure that it was a single lysogen. β-Galactosidase assays of lysogens resulted in a basal average activity or a multiple of that average. We assumed that the lowest value was that produced from a single prophage. To support this assumption, we infected potential single and double lysogens with λC17*c*I$^-{}_{90}$, which has the ability to produce virulence in combination with certain *cis c*I mutations (1, 20, 25). When a single lysogen containing a single copy of the repressor gene is infected with λC17*c*I$^-{}_{90}$, it will fail to be immune to the phage at a certain multiplicity of infection, whereas at the same multiplicity a double lysogen will be immune, since two copies of the repressor gene are available. The lysogens were grown in 5 ml of TBM medium overnight, centrifuged at 5,000 × *g* for 5 min, and suspended in 2 ml of 10 mM $MgSO_4$. The cells were plated in TB top agar, and several concentrations of λC17*c*I$^-{}_{90}$ were spotted onto TB plates. The plates were incubated at 37°C for 6 to 8 h. A single lysogen will lyse cells, indicated by a clear spot on the lawn, faster than a multiple lysogen will, since single lysogens are more sensitive to λC17*c*I$^-{}_{90}$.

An additional test for single lysogens involved preparing a low-titer lysate on potential single and double lysogens (23). A low-titer lysate was obtained by growing lysogens overnight in LB-ampicillin (5 ml) and then spinning down cells by centrifugation at 5,000 × *g* for 5 min. The supernatant fraction, which was the low-titer lysate, was titered as described by Silhavy et al. (23). A single lysogen produces a substantially lower titer lysate than does a double lysogen in this test.

In all cases, the lysogens with the lowest β-galactosidase activities were the most sensitive to λC17*c*I$^-{}_{90}$ infection and had the lowest titers. These results indicated that the β-galactosidase activity values reported most likely represented those obtained from single lysogens.

Specific activity assays. To ensure that differences in β-galactosidase activity between the fusions were due to different levels of β-galactosidase protein and not to different specific activities of the hybrid protein fusions, we measured the amount of β-galactosidase protein present in the extracts from lysogens by immunostaining cell extracts with anti-β-galactosidase antibodies. The protein concentrations, as determined by the Coomassie brilliant blue assay (2), were adjusted to give equal amounts of protein, and serial dilutions of each extract were spotted onto nitrocellulose and dried. The subsequent immunostaining procedure was carried out by using a kit (Protoblot) and other reagents from Promega Biotek (Madison, Wis.). The nitrocellulose blot was treated with TBST (10 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 1% Blot-Qualified bovine serum albumin for 30 min to block all unbound protein-binding sites. The blot was then incubated for 30 min at room temperature with mouse anti-β-galactosidase antibody, followed by three 10-min rinses in TBST with continual agitation. The blot was then treated with anti-mouse immunoglobulin G conjugated to alkaline phosphatase for 30 min at room temperature and then again rinsed three times with TBST. Color development was initiated by placing the blot in a substrate solution consisting of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris hydrochloride (pH 9.5)–100 mM NaCl–5 mM $MgCl_2$. After the color had developed to the desired intensity, the reaction was stopped by rinsing the blot in deionized water.

Assays of fusion protein stabilities. The stabilities of fusion proteins were assayed on cells carrying the fusion plasmids. Cells were grown to an OD_{600} of 1 in 10 ml of minimal A medium, treated with a 15-min pulse of 50 μCi of [^{35}S]methionine (1,000 Ci/mol; Dupont, NEN Research Products, Boston, Mass.), and chased with an excess of unlabeled methionine. At 0, 10, 20, and 30 min after addition of chase, 2.5-ml portions were removed; after being pelleted by centrifugation, the cells were dissolved in 83 μl of SDS sodium dodecyl sulfate loading buffer (0.5 ml of β-mercaptoethanol, 0.25 ml of 0.1% bromophenol blue, 4 ml of 10% SDS, 5.3 ml of 2× sample buffer [0.25 M Tris hydrochloride {pH 6.8}, 0.2% SDS, 20% glycerol]) as described previously (23). The proteins were separated on 10% SDS-polyacrylamide gels and subjected to autoradiography to localize fusion proteins.

RESULTS

Construction of *lacZ* fusion plasmids. In-frame fusions between *uncI*, *uncB* or *uncE*, and *'lacZ* were constructed as described in Materials and Methods (Fig. 1). All of the fusions contained the *unc* promoter so that they could be transcribed from the true promoter of the *unc* operon when they were crossed into the chromosome at the *att* site. Four different *uncIB'-'lacZ* fusions were made to test the effect of the amount of *uncB* DNA fused to *'lacZ* on β-galactosidase activity.

Measurements of *uncB* and *uncE* gene expression. Fusion genes were moved from multicopy plasmids into the chromosome of *E. coli* MC1000Δ(*uncI-uncC*), where each would be present in single copy, to compare activities between fusions in different genes and to determine relative expression. The λ transducing phage λRZ5 was used to transfer the gene fusions from the plasmids to the *att* site in the chromosome.

Table 2 shows the results obtained from β-galactosidase assays of the various lysogens. The amount of β-galactosidase activity produced from cells lysogenized with a single copy of the early *uncB'-'lacZ* fusions B_1, B_2, and B_3, which contain, respectively, 60, 92, and 126 bases of *uncB*, was comparable to that produced from cells containing the *uncE'-'lacZ* fusion, although cells carrying the B_3 fusion did appear to produce about twofold-higher activity than did cells carrying the other three fusions. Cells lysogenized with a single copy of the late *uncB'-'lacZ* fusion B_4, which contains 704 base pairs of *uncB*, produced approximately 10 times less β-galactosidase activity than did cells containing the *uncE'-'lacZ* fusion or the other three *uncB'-'lacZ* fusions. The relative expression of 1:10 for *uncB* and *uncE* agrees with the ratio measured in purified F_1F_0 preparations (7).

Measurement of *uncI* gene expression. Lysogens carrying the *lacZ* fusion in *uncI* were assayed for β-galactosidase activity (Table 2). The measured activity obtained from cells lysogenized with the *uncI'-'lacZ* gene fusion was not significantly different from background levels of β-galactosidase activity obtained from MC1000Δ(*uncI-uncC*) alone. A 30-h incubation of the lysogen lysate with substrate produced more color than did the control assay, but the calculated activity was well below 1 U. Therefore, a single copy of *uncI* in the chromosome was expressed very poorly, at best 10- to 20-fold less well than *uncB*.

We also measured β-galactosidase activity in MC1000Δ(*uncI-uncC*) cells containing the *uncI'-'lacZ* fusion plasmid pKS101. Surprisingly, when the *uncI* fusion was present on a multicopy plasmid, the level of expression from the plas-

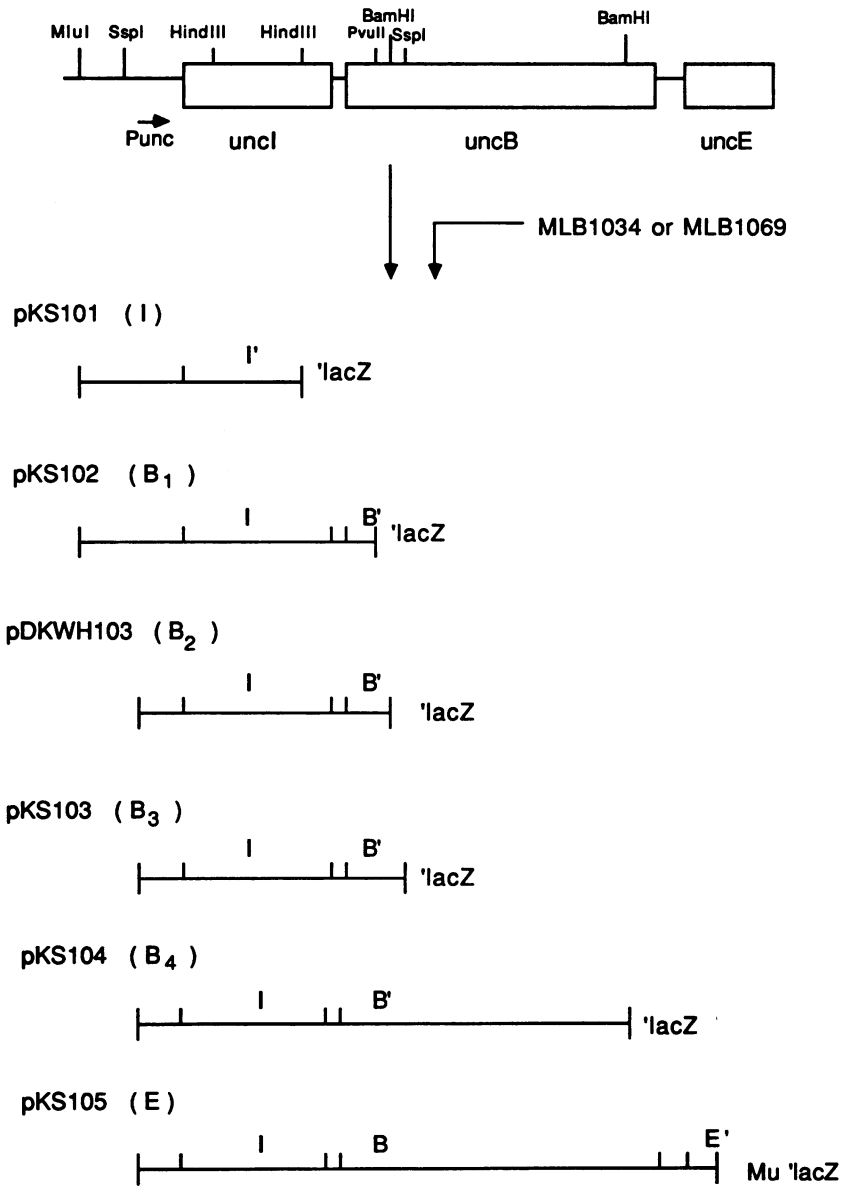

FIG. 1. Fusion plasmids constructed for this study. The upper line indicates restriction sites present in the region preceding the *unc* promoter as well as in *uncI*, *uncB*, and *uncE* of the *E. coli unc* operon. Only restriction sites used for the constructions are shown, and spacing between the sites is approximate; exact spacing is given in Materials and Methods. The limits of the *unc* genes are indicated. The designation for each plasmid is followed by a line indicating the limits of the *unc* DNA fused to *'lacZ*. Plasmid pKS105 contains 117 base pairs of phage Mu DNA in addition to *'lacZ*.

mid was 16 times higher than in cells containing the control plasmid pMLB1069 (48 U versus 3 U). The *uncI* gene appeared to be expressed relatively better when present on a multicopy plasmid than when present in a single copy in the chromosome. The level of expression from a multicopy plasmid, however, was still much lower than the expression of any of the other *unc-'lacZ* fusion genes present on a multicopy plasmid, which typically produced several thousand units of activity in this assay.

Tests of specific activity and stability of the fusion proteins. Differences in measured β-galactosidase activities might be due to differences in gene expression or to different specific activities or stabilities of the fusion proteins. Although it has been previously observed that the specific activities of protein fusions containing different amounts of protein fused to β-galactosidase change very little (8), studies on *lac* fusions to *malF* suggest that the specific activity of a fusion to membrane-bound protein depends on the topology of the fusion protein (9). Different specific activities could account for the differences in activities obtained from the gene fusions, in particular the very low activities produced by the I and the B_4 fusions in comparison with the B_1, B_2, B_3, or E fusion. We therefore measured the relative specific activity and relative stability of each fusion protein in order to draw conclusions about relative rates of expression of genes from measurements of β-galactosidase activity.

The specific activity of each fusion protein was assayed by carrying out an immunostain of serial dilutions of cell extracts containing each fusion and testing for reactivity with anti-β-galactosidase antibodies. In all cases, the specific

TABLE 2. β-Galactosidase activities obtained from MC1000Δ(*uncI-uncC*) lysogenized with the λ transducing phage containing the *uncI'-*, *uncB'-*, and *uncE'-'lacZ* fusions

Transducing phage	Mean activity[a] (β-galactosidase activity units) ± SEM
MC1000Δ(*uncI-uncC*)	0
λpKS101 (I)	<1
λpKS102 (B_1)	170 ± 10
λpDKWH103 (B_2)	127 ± 3
λpKS103 (B_3)	300 ± 8
λpKS104 (B_4)	15 ± 3
λpKS105 (E)	166 ± 9

[a] Assays were carried out as described in Materials and Methods. The mean was obtained by averaging values obtained from 8 to 10 different lysogens.

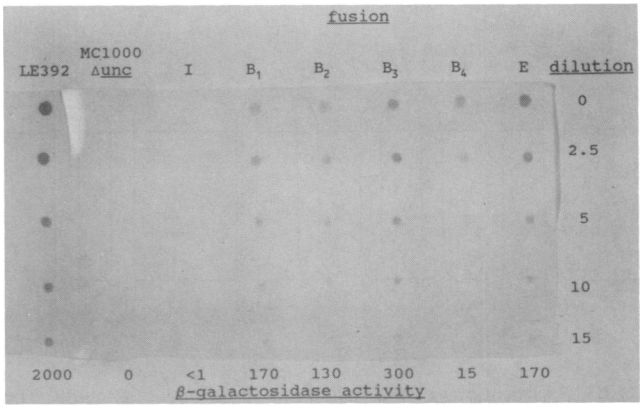

FIG. 2. Estimation of specific activities by immunostain. Cell lysates from lysogens (listed at the top) were prepared, spotted, and stained as described in Materials and Methods. The controls were LE392 (*lac*$^+$) and MC1000Δ*unc* (Lac$^-$). The average β-galactosidase activities calculated for the different sets of fusion-bearing lysogens are indicated at the bottom and are identical to the activities reported in Table 2. Actual activities produced by the lysogens selected for in this experiment were within the standard error of the mean for each set. Dilutions of lysates represented by each row of spots are given on the right.

activities of the fusion proteins were the same within a factor of 2 (Fig. 2). These results indicated that the differences in amount of β-galactosidase activity obtained from the fusions were not due to differences in the amount of ATPase protein fused to β-galactosidase.

The stability of each of the fusion proteins was also measured to determine how rapidly these proteins were turned over, since differences in protein stability may also have an effect on the amount of β-galactosidase activity obtained from each of the fusion genes. Pulse-chase labeling experiments on cells carrying the fusion plasmids were carried out as described in Materials and Methods. The experiments showed that even after a 30-min chase, the amounts of the *uncI*, *uncB*, and *uncE-lacZ* fusion proteins did not change (not shown). Therefore, large differences in β-galactosidase activities cannot be attributed to differences in turnover rates of the fusion proteins, since the stabilities of all of the fusion proteins were very similar if not the same. These controls indicated that the differences in β-galactosidase activities were caused by differences in gene expression.

DISCUSSION

The unusual stoichiometry of the a and c subunits in the assembled ATPase complex (a_1:c_{10}) raises questions about the regulation of gene expression within the *unc* operon. If the stoichiometry of subunits in the complex reflects the rates of expression of the corresponding genes, then the synthesis of these subunits from the polycistronic mRNA must increase as translation proceeds from *uncB* to *uncE*. Direct measurements of in vivo expression of a gene can be achieved by using *lacZ* protein fusions (reviewed in reference 24). We constructed *lacZ* fusions in *uncB* and *uncE* to determine whether these genes are expressed differently in vivo and whether the relative levels of expression correspond to the numbers of these subunits assembled into the ATPase complex. The in vivo expression of *uncI*, which codes for the 14-kilodalton i protein, was also measured.

There are three possible reasons for observing different levels of β-galactosidase activity from different *lac* fusions. The first is that the fusion genes are differentially expressed, and the different levels of β-galactosidase activity accurately reflect this differential expression. If the genes are not differentially expressed, the second and third possibilities for different activities are significantly different stabilities or specific activities for the resultant fusion proteins. It has been shown, for example, that *lac* fusions to the *E. coli malF* gene can produce fusion proteins with dramatically lowered specific activities, perhaps caused by the topology of the membrane-bound fusion protein (9). We therefore tested both the stabilities and the specific activities of our fusion proteins. Our pulse-chase studies revealed that after a 10-min pulse of [^{35}S]methionine, in a 30-min chase there was no significant turnover of any of these fusion proteins. Therefore, the large (greater than 10-fold) differences in activities produced by the I or B_4 fusion compared with activities of the others cannot be accounted for by different stabilities.

To control for different specific activities, we carried out immunoblots of serial dilutions of cell extracts from the lysogens, testing for anti-β-galactosidase-reactive material. In this experiment, differences in reactivity to anti-β-galactosidase should provide an accurate measurement of differential production of the fusion proteins regardless of specific activities. If the differences seen in the immunostain are the same as the differences in enzyme activities, the specific activities of the fusion proteins are the same. The results demonstrated clearly that there were not large differences in the specific activities of any of the fusion proteins. The specific activity of the B_4 fusion might be slightly lower than the specific activities of the other B fusions, but not by more than a factor of 2. Also, the specific activity of the E fusion appeared to be slightly lower than that of the B_1, B_2, or B_3 fusion. We conclude from these control experiments that the different levels of β-galactosidase activity produced by the different gene fusions constructed for this study do reflect different levels of gene expression.

The results indicated that *uncB* and *uncE* were expressed differently in vivo. Comparison of the β-galactosidase activities produced by the lysogens carrying the B_4 fusion with the activity produced by the lysogen carrying the E fusion demonstrated an a:c ratio of approximately 1:10, which is the ratio determined for the a and c subunits in the purified F_1F_0 ATPase complex (7). Figure 3 shows two-dimensional models for the organization of the a and c subunits in the cytoplasmic membrane of *E. coli*. Although this figure shows that the early B fusions produced proteins containing a portion of the N terminus that might normally be found in the periplasm, these fusion proteins do not contain any of the putative membrane-spanning regions and are therefore prob-

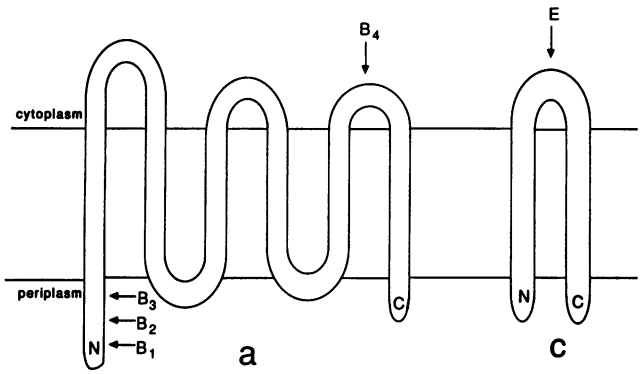

FIG. 3. Locations of *lac* fusions to the a and c subunits. The two-dimensional structure of the a subunit is taken from reference 27, and that of the c subunit is taken from reference 21. ↓, Approximate locations of fusion joints in the *unc'-'lacZ* fusion proteins produced from λpKS102 (B_1), λpDKWH101 (B_2), λpKS103 (B_3), λpKS104 (B_4), and λpKS105 (E). Each fusion protein contains the subunit from the N terminus to the point indicated by the arrow. Although the B_1, B_2, and B_3 fusion proteins contain a part of the a subunit which this figure shows to be periplasmic, it is most likely that all three fusion proteins are located in the cytoplasm, since none contain any of the putative membrane-spanning sections.

ably present in soluble forms in the cytoplasm. The B_4 protein fusion joint is in a section of the a subunit that is believed to be facing the cytoplasm, connecting the last two of six putative transmembrane hydrophobic stretches (27). This fusion protein contains 86% of the a subunit. The E-protein fusion joint is also in a cytoplasm-facing section of the c subunit which connects the two presumed transmembrane sections, and the fusion protein contains 53% of the c subunit. Since both of these fusion proteins contain more than half of their respective ATPase subunits, and since they have similar topologies, stabilities, and specific activities, a comparison of their activities should accurately measure the differences in in vivo expression of *uncB* and *uncE*.

It is not clear why the early B fusions, B_1, B_2, and B_3, are expressed so much better than the late B fusion, B_4. Perhaps the twofold-higher activity produced from the B_3 fusion (Table 2) can be accounted for by the possible error in our measurements of stability or specific activity. However, the extent of that error cannot account for the 10- to 20-fold differences seen in the activity produced from the B_1, B_2, or B_3 fusion compared with the activity produced from the B_4 fusion. The former set of fusion genes appears to be expressed substantially better than the latter. They are expressed as well as the *uncE'-'lacZ* fusion gene *E*. A similar phenomenon has been observed for early and late fusions to the *E. coli malF* gene (9).

This difference in expression of early and late gene fusions could be due to either transcriptional or translational effects. Studies on the *unc* transcript, however, revealed no significant processing (14), so it is unlikely that the three early *uncB'-'lacZ* fusion genes are transcribed better than the late fusion. It is more likely that the early fusions are translated better than the late fusion and that signals exist within *uncB* which slow down the ribosomes as they are translating. Those signals might be mRNA secondary structures, rare codons, or perhaps even changes in translational rates resulting from cotranslational insertion of the fusion protein into the cytoplasmic membrane.

These experiments also measured the level of expression of *uncI*, the first gene in the *unc* operon. The *uncI* gene is an open reading frame which, if transcribed and translated, would produce a protein with a molecular weight of 14,200. A protein of this size has been shown to be synthesized from in vitro transcription-translation studies of cloned *uncI* DNA (5). Results from our studies indicate that *uncI* is essentially not expressed in vivo under conditions of aerobic growth in defined medium. When the *uncI'-'lacZ* gene fusion was present in a single copy in the chromosome, the amount of β-galactosidase activity produced was only slightly higher than background levels. The in vivo expression of this gene is therefore at best 10 to 20 times lower than the in vivo expression of the B_4 gene, which is expressed at levels comparable to the in vivo level of expression of *uncG*, which we have previously quantitated (1). The i protein is therefore not produced in stoichiometric amounts in comparison with amounts of the ATPase subunits. Other studies have shown that insertions and deletions within *uncI* do not affect ATPase activity (11, 28). In light of our studies on in vivo gene expression, those results are not surprising. If the i protein is involved in the synthesis, assembly, or activity of the ATPase, its role does not appear to be structural.

We also measured the β-galactosidase activity from the *uncI'-'lacZ* fusion when the fusion was present on a multicopy plasmid. Interestingly, on a plasmid, the level of *uncI* expression was easily measurable and was about 16 times higher than the control level. Whether the difference between low-copy expression of *uncI* and high-copy expression of *uncI* is a function simply of copy number or of some control mechanism remains to be determined.

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